Partial Characterization of a Cell Motility Factor from Human Urine

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ABSTRACT

Dialyzed, concentrated urine from 21 patients with a history of bladder cancer or a gross bladder tumor was tested for cell motility activity using BALB/c/3T3 cells. Thirteen urine samples from patients with a gross bladder tumor produced a greater increase in cell migration than 8 urine samples from patients with a history of bladder cancer [167% ± 14 (S.E.), 64% ± 19, respectively; p < 0.001]. Protease treatment of urine from a patient with bladder cancer caused a 95% loss of activity, while heating to 100° for 2 min caused an 86% loss of activity. High-performance liquid chromatography of urine from a patient with bladder cancer revealed that the greatest activity was present in fractions with a molecular weight between 18,000 and 30,000. These results suggest that motility-stimulating factor may be a useful marker for detecting carcinoma of the bladder.

INTRODUCTION

Superficial carcinoma of the bladder is a disease which recurs in approximately 70% of patients. Currently, the most widely used noninvasive method of detection is urine cytology which, unfortunately, has a low sensitivity for well-differentiated tumors (13). As a result, it cannot be used as a screening test for this disease. Recently, Chodak et al. (5) used an in vitro assay to measure capillary endothelial cell motility and showed that urine from patients with transitional cell carcinoma contained significantly greater motility activity than urine from patients with benign disease or a history of bladder cancer. When a tumor was removed, the activity decreased significantly and then increased again with the appearance of another tumor. This work suggested that cell motility could potentially be used as a marker for detecting this disease. This motility assay is difficult to perform because endothelial cells cannot easily be maintained in culture. Evidence that 3T3 cells respond to several growth factors (6, 8, 12) raised the possibility that these cells might also respond in a motility assay.

We now report on the partial characterization of a factor in urine which causes cell motility of bovine capillary and BALB/c/3T3 cells. Our results suggest that this response is caused by a heat-labile, protease-sensitive macromolecule with a molecular weight between 18,000 and 30,000.

MATERIALS AND METHODS

Growth of BALB/c/3T3 Cells. BALB/c/3T3 cells (clone A31) originally prepared by Aaronson and Todaro (1) were maintained by feeding with Dulbecco’s Eagle’s medium supplemented with glucose (4.5 g/liter; Grand Island Biological Co.), 10% calf serum (Colorado Serum Co.), and 50 microm of penicillin per ml and 50 μg of streptomycin per ml. The cells were passaged before reaching confluence in order to maintain the property of contact inhibition. For the assay, however, a flask of cells was permitted to reach confluence. In preliminary experiments, we found that confluent cells fed 4 to 6 days prior to the assay resulted in the lowest background motility for control cultures.

Urine Collection. Urine collection, storage, and processing have been described previously (5). The only modifications were a change in the molecular weight cutoff of the dialysis tubing to 6000 to 8000, and the use of a YM5 ultrafiltration filter (M, 5000; Amicon Co.). The presence or absence of a tumor was documented by a cystoscopic examination and a biopsy when a gross tumor was evident.

3T3 Cell Motility Assay. The cell motility assay as designed by Albrach-Buehler (2, 3) and modified by Zetter (14) has been described previously (5). The confluent 3T3 cells used for the migration assay were trypsinated to create a single-cell suspension, and the trypsin was inactivated by adding Dulbecco’s modified Eagle’s medium that contained 2% calf serum. Three thousand cells were added to 30-mm dishes containing a gelatin-coated coverslip, and the cultures were incubated for 90 min before the urine samples were added. Four different volumes of urine were tested that included 5, 10, 25, and 50 μl. The tracks produced by the cells were projected onto a television screen and a digital image analyzer (Numonics Corp.) was used to determine the mean area of motility of 81 cells for each dish (Fig. 1). Control cultures containing only cells and culture media were performed in triplicate. To determine the percentage increase in cell motility, the following formula was used:

\[
\text{% of increase in cell motility} = \frac{\text{Mean area movement for urine sample}}{\text{Mean area of movement for controls}} \times 100\%
\]

This calculation was made to account for variations in the cell response from day to day. For each sample, the maximum percentage increase in migration was selected from among the 4 urine volumes tested, and this value was used to compare to other samples.

BCE Cell Motility. The growth and maintenance of BCE cells, as well as the motility assay, has been described previously (14). These cells were used for assessing motility in the fractions generated from HPLC, and the percentage increase in cell motility was again calculated.

Heat Stability. To assess the effect of heat on the motility-stimulating factor, a dialyzed and concentrated urine specimen from a patient with bladder cancer was divided into 4 equal volumes. One sample was maintained at 4°, while the remaining 3 were subjected to the following conditions; one sample was incubated at 37° for 1 hr, one at 56° for 30 min, and the last sample was heated to 100° for 2 min. Following exposure to the test conditions, each sample was centrifuged, and the supernatants were tested in the motility assay. Duplicate samples were tested at each temperature, and the entire experiment was repeated using urine from another patient with bladder cancer. The percentage of motility-stimulating activity which remained after each treatment was calculated by comparing each sample to the sample maintained at 4°.

The abbreviations used are: BCE, bovine capillary endothelial; HPLC, high-performance liquid chromatography.
This was calculated according to the following:

\[
\% \text{ of activity remaining} = \frac{\text{Mean area of cell movement at test temperature}}{\text{Mean area of cell movement at } 4^\circ} \times 100\%
\]

Protease Treatment. Dialyzed and concentrated urine from a patient with bladder cancer was titrated to pH 7.5 with 0.1 N NaOH and then divided into 5 aliquots of 1 ml each. Trypsin (Sigma Chemical Co.; 11,000 units/mg) and chymotrypsin (Sigma; 43 units/mg) were dissolved in 0.03 M Tris/0.15 M CaCl buffer to make a 25-mg/ml solution of each compound. The trypsin and chymotrypsin mixture was added to one of the urine aliquots to make a final concentration of 2500 µg of trypsin and 2500 µg of chymotrypsin per ml of urine. The mixture was incubated for 90 min at 37°, and then the proteases were inactivated by adding a 5-fold weight excess of soybean trypsin inhibitor in Tris/CaCl buffer.

Four controls were performed simultaneously: one contained water and urine but no proteases or inhibitor, another contained only urine and buffer, the third contained urine, protease inhibitor, and inactive proteases, and the fourth contained urine, buffer, and trypsin inhibitor but no proteases. The inactive proteases were prepared by boiling equal volumes of trypsin and chymotrypsin together for 30 min at 100°, centrifuging the mixture at 2000 rpm for 2 min, and using the supernatant for the experiment.

Ten and 100 µl of each of the 5 mixtures were tested in duplicate in the motility assay. The percentage of migration activity remaining in each aliquot was calculated by the following formula using the activity in the sample containing urine and water as the reference:

\[
\% \text{ of activity remaining} = \frac{\text{Mean area cell motility in test sample}}{\text{Mean area cell motility of untreated urine}} \times 100\%
\]

The entire experiment was repeated, and the results reflect an average of the 2 assays.

HPLC. A urine sample from a patient with documented bladder cancer and one from a patient with benign disease were dialyzed and concentrated as described earlier and then frozen and lyophilized. Five mg of each lyophilized sample were dissolved in 0.1 M ammonium sulfate/0.05 M sodium phosphate buffer (pH 7.0) and analyzed on a Beckman Model 332 HPLC System using HPLC TSK 2000 columns. The flow rate was 1.0 ml/min and 0.8-ml fractions were collected at room temperature. The column was equilibrated with ovalbumin (M, 43,000), myoglobulin (M, 17,800), and insulin (M, 5,800). The percentage of increase in 3T3 and BCE cell motility was assessed in 50 µl of each fraction.

RESULTS

Motility Activity in Urine from Patients with a Gross Bladder Tumor. Thirteen urine samples collected from 11 patients with transitional cell carcinoma, one patient with a squamous cell carcinoma, and one patient with an adenocarcinoma of the colon metastatic to the bladder were tested for cell motility activity using BALB/c/3T3 cells. The percentage of increase in cell motility over background ranged from 65 to 262% with a mean of 167% (±14%; Chart 1). Only one patient produced less than a 110% increase in cell motility. There was no apparent correlation between tumor grade and the level of activity.

Motility-stimulating Activity in Urine from Patients with a History of a Bladder Tumor. Urine samples collected from 8 patients with a recent or past history of a bladder tumor were tested for motility activity, and the level ranged from 13 to 190% with a mean increase of 64% (±19%; Chart 1). This level was significantly lower than the mean for the group with a gross tumor (p < 0.001 by Student's t test). Urine from only one patient stimulated more than 110% increase in cell movement compared to controls. This patient had previously undergone a nephroureterectomy followed 2 years later by a cystectomy, both for transitional cell carcinoma. The urine used in this study was collected from the ileal conduit. Two months after the urine was tested for activity, the patient was found to have metastatic bladder cancer.

Characterization of Motility-stimulating Activity. Urine from a patient with a bladder tumor was incubated at 4 different temperatures prior to testing in the cell motility assay. Increasing the temperature resulted in substantial reduction of activity (Chart 2). Compared to urine incubated at 4°, urine heated to 37° for 1 hr lost 18% of motility-stimulating activity, while heating to 56° for 30 min resulted in a loss of 35% of the activity. Following boiling for 2 min, a precipitate developed which was toxic to the cells. When only the supernatant was assayed, 86%
of the activity was lost. It is entirely possible that activity could have been retained in the precipitate. However, even at 56°C, some motility activity was lost.

Protease digestion also affected motility activity. The addition of trypsin and chymotrypsin to urine from a patient with a bladder tumor resulted in a 95% loss of activity compared to the untreated urine (Chart 3). In contrast, when heat-inactivated proteases were used, no activity was lost. Urine containing either trypsin inhibitor or buffer alone stimulated cell motility similar to the control which contained only urine and water.

To obtain some information about the molecular weight of the factor responsible for migration, HPLC was performed on a urine sample from a patient with a bladder tumor and on urine from a patient with no disease. Each fraction was tested for cell motility, and the most activity was present in a fraction corresponding to a molecular weight between 18,000 and 30,000 (Chart 4). Several peaks of activity were noted in this molecular weight range. No significant activity was detected in any fraction derived from normal urine (Chart 4).

To determine the similarity between migration of 3T3 cells and capillary cells, each cell was used to test for activity in fractions obtained following HPLC of a urine sample from a patient with bladder cancer. Similar peaks of activity were obtained with the 2 cell lines ranging between $M_r$ 18,000 and 30,000 (Chart 5).

**DISCUSSION**

Over the past several years, angiogenesis activity has been studied as a potentially new marker for identifying tumors of the eye (11) and central nervous system (9). Based on the observation that angiogenesis consists of capillary endothelial cell migra-
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tion and proliferation (4). Chodak et al. (5) reported that a cell motility assay could be used to detect the presence of a gross bladder tumor (5).

We now report the first partial characterization of this cell motility activity from human urine obtained from a patient with bladder cancer. Cell motility appears to be caused by a heat-labile protein macromolecule with the major activity having a molecular weight between 18,000 and 30,000.

This work is significant because it further suggests that urine may be tested for this substance and eventually be used to detect carcinoma of the bladder. This will probably require the development of a radioimmunoassay, and characterization and purification are a necessary first step. Although cell motility factors are present in other body fluids bathing tumors, urine offers 2 important advantages as a source of material for the purification process: it readily can be obtained in large quantities, and invasive methods are not required. Both should help facilitate progress in this area.

Another factor which may facilitate purification is the demonstration that BALB/c/3T3 cells as well as capillary endothelial cells can be used as target cells to measure activity. The 3T3 cells are useful because they are easier to maintain and because unstimulated 3T3 cells have a lower background of motility compared to unstimulated capillary endothelial cells. Other investigators have shown that 3T3 cells respond to several growth factors (6, 8, 12). Although presently there is no conclusive evidence that the tumor factor which stimulates endothelial cells is the same factor which stimulates 3T3 cells, several observations suggest this is the case. First, in this study, HPLC of urine from a patient with bladder cancer resulted in factors with similar peaks of activity as measured by both 3T3 cells and capillary endothelial cells. In addition, recent work by Shing et al. (10) revealed that a factor derived from a chondrosarcoma is a mitogen for both cell types. Further studies are needed, however, to conclusively show the same factor stimulates both cell types.

One question that arises from this work is whether epidermal growth factor is the substance in urine which stimulates cell motility. One observation suggests that this is unlikely. In both this study and the one by Shing et al. (10), the molecular weight of the tumor product was 17,000. In contrast, epidermal growth factor has a molecular weight of only 6000, although Hirata and Orth (7) have shown that epidermal growth factor may have components in the M, 28,000 to 33,000 range. Future studies will be directed at determining the relationship between the tumor-induced factor in human urine and epidermal growth factor.

Another finding from this study deserves comment. One patient with an ileal conduit had an elevated level of motility activity shortly before presenting with metastatic disease. Three possible explanations may be offered; either the conduit results in high motility activity, the patient had upper-tract disease, or motility activity from metastatic disease passed into the urine. In our previous study, patients with an ileal conduit and no evidence of cancer had low levels of BCE cell motility activity which means that this is not a likely explanation. In this case, the patient did not have evidence of upper-tract disease. This leaves the third possibility that motility activity from metastases passes into the urine. This intriguing idea warrants further investigation.

In summary, this study describes the first partial characterization of a cell migration factor in human urine obtained in the presence of a gross bladder tumor. Further investigation and purification are now under way in order to determine if a diagnostic assay can be developed based on this cell motility factor.

REFERENCES

Fig. 1. Photomicrograph of 3T3 cells grown on glass slides covered with gold particles. Cells incubated for 18 hr in urine from patients with bladder tumor (A) demonstrate greater cell motility (more gold ingestion) than cells incubated in media alone (B). x 40.
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